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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:
MANOSROI *et al.*
Appl. No. 09/987,457
Filed: November 14, 2001
For: **Methods for Large Scale Protein Production in Prokaryotes**

Confirmation No. 6677
Art Unit: 1632
Examiner: To be assigned
Atty. Docket: 0652.2180001/EKS/Y-W

Submission of Substitute Certified Copy of Priority Document

Commissioner for Patents
Washington, D.C. 20231

Sir:

A certified copy of the priority document GB 00 27 782.2 was submitted on November 14, 2001 for the above-captioned application in good faith. However, it has now been discovered that page 18 of the submitted certified copy was accidentally missing. Applicants therefore respectfully submit herewith a complete substitute certified copy of the priority document GB 00 27 782.2. Page 18 is simply a page of references and contains no new matter. Applicants respectfully request that the United States Patent and Trademark Office excuse this error and replace the incomplete copy with the substitute certified copy of the priority document submitted herewith.

Respectfully submitted,

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Date: 6/17/02

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2.	**Patent application number** *(The Patent Office will fill in this pa*	**0027782.2**
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	Patents ADP number *(if you know it)*	00342931001
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4.	**Title of the invention**	Methods for large scale protein production in prokaryotes
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Methods for large scale protein production in prokaryotes

The invention belongs to the field of protein production in prokaryotic cells.

The invention relates to methods for the production of recombinant DNA-derived heterologous protein in prokaryotic cells, wherein said heterologous protein is secreted extracellularly as an active and correctly folded protein, and the prokaryotic cell contains and expresses a vector comprising the DNA coding for said heterologous protein operably linked to the DNA coding for the signal peptide OmpA.

Background art

Prokaryotic expression systems for heterologous proteins are commonly used for proteins which do not require mammalian glycosylation patterns as they provide a cheap way of producing large quantities of said protein. The formation of highly aggregated protein or inclusion bodies can be commonly found in high-level expression of many heterologous proteins in *E.coli* . One way of protein production is via inclusion bodies which develop in cytoplasm. Cell wall and outer membrane components of the prokaryotic cells used for production (e.g. *E.coli)* usually contaminate the cell lysate containing the heterologous protein when said inclusion bodies are prepared by low-speed centrifugation. The outer membrane component can be eliminated by selective extraction with detergents and low concentrations of either urea or guanidine·HCl.

One example of such a heterologous protein is a tPA derivative.

Tissue plasminogen activator (tPA) is a polypeptide containing 527 amino acid residues (27) with a molecular mass of 72 kDa. The molecule is divided into five structural domains. Nearby the N-terminal region is a looped finger domain, which is followed by a growth factor domain. Two similar domains, kringle 1 and kringle 2, are following. Both finger and kringle 2 domains bind specifically to the fibrin clots thereby accelerating tPA protein activation of bound plasminogen. Downstream of kringle 2 is the serine protease, with its catalytic site located at the C-terminus. The serine protease is responsible for converting plasminogen to plasmin a reaction important in the homeostasis of fibrin formation and clot dissolution. The correct folding of tPA requires the correct pairing of 17 disulfide bridges in the molecule (1).

Clinically, tPA is a thrombolytic agent of choice for the treatment of acute myocardial infarction. It has the advantage of causing no side effects on systemic haemorrhaging and fibrinogen depletion (7). Bowes melanoma cells were first used as a source in tPA production for therapeutic purposes (12). Since a consistent process with efficient production of highly purified protein in good yield is required for clinical use, the construction of full-length recombinant-tPA (r-tPA) progressed to mammalian cells. Chinese hamster ovary cells were transfected with the tPA gene to synthesize the r-tPA (8, 22). The recombinant product produced by a mammalian fermentation system was harvested from the culture medium. Attracted by simplicity and economy of production, a number of efforts in producing r-tPA from bacteria, especially from *Escherichia coli*, were investigated (10, 13, 30). Regarding the low yield and the formation of inclusion bodies, which resulted in misfolding and in an inactive enzyme, numerous strategies have been proposed to overcome these problems. The major criterion is to synthesize the smallest molecule, which is still active instead of full-length tPA.

Several deletion-mutant variants including kringle 2 plus serine protease (*K2S*) were considered. However, the enzymatic activity of the recombinant-K2S (r-K2S) was obtained only when refolding processes of purified inclusion bodies from cytoplasmic compartment were achieved (16, 29). In order to avoid the cumbersome refolding processes and periplasmic protein delivery, special bacterial expression systems were exploited (6, 31). Despite periplasmic expression of tPA, overexpression led to inactive aggregates, even in the relatively high oxidizing condition in the periplasm.

In the prior art, there are a few descriptions of methods for the preparation of recombinant K2S in E. coli. However, there is no disclosure of a method leading to a cost effective method for large scale production of biologically active K2S.

Obukowicz et al. (25) expressed and purified r-K2S from periplasmic space. The obvious disadvantage of this method was an extra periplasmic extraction step, which is not suitable for large scale production.

Saito et al. (29) disclose the cytoplasmic expression of r-K2S. The authors used an in vivo renaturation processes for the expressed r-K2S, which was purified from the cytoplasmic space of E. coli as inclusion body. Boehringer Mannheim use a similar cumbersome denaturing/refolding process involving the steps of cell digestion, solubilization under denaturing and reducing conditions and reactivation under oxidizing conditions in the

presence of GSH/GSSG which is not cost effective and requires mutation of the amino acid sequence (24).

In 1991, Waldenström et al. (34) constructed a vector (pEZZK2P) for the secretion of kringle 2 plus serine protease domain to E. coli culture supernatant. Hydroxylamine was used to remove the ZZ fusion peptide from IgG-Sepharose purified fraction. The cleavage agent hydroxylamine required modification of the cleavage sites of kringle 2 plus serine protease (Asn^{177} → Ser and Asn^{184} → Gln) thus to protect it from hydroxylamine digestion. However, the resulting non-native, not properly folded K2S molecule is not suitable for therapeutic purposes. The unusual sequence may even activate the human immune system.

The problem underlying the present invention was thus to provide a commercially applicable method for large scale production of heterologous proteins, e.g. K2S, wherein the heterologous protein is secreted in its biologically active form into the culture supernatant.

Description of the invention

The problem was solved within the scope of the claims and specification of the present invention.

The use of the singular or plural in the claims or specification is in no way intended to be limiting and also includes the other form.

The invention relates to a method for the production of recombinant DNA-derived heterologous protein in prokaryotic cells, wherein said heterologous protein is secreted extracellularly as an active and correctly folded protein, characterized in that the prokaryotic cell contains and expresses a vector comprising the DNA coding for said heterologous protein operably linked to the DNA coding for the signal peptide OmpA or a functional derivative thereof.

Surprisingly, the use of the signal peptide OmpA alone and/ or in combination with the N-terminal amino acids SEGN/SEGNSD translocate the recombinant DNA-derived proteins to the outer surface and facilitates the release of the functional and active molecule into the culture medium to a greater extent than any other method in the prior art. Before crossing the outer membrane, the recombinant DNA-derived protein is correctly folded according to the method of the present invention. The signal peptide is cleaved off to produce a mature molecule. Surprisingly, the efficiency of signal peptide removal is very high and leads to correct folding of the recombinant DNA-derived protein. This method according to the

invention, exemplified for the kringle 2 plus serine protease domain (K2S) of tissue plasminogen activator protein in example 1 is generally applicable to expression of several different proteins and polypeptides which do not require mammalian glycosylation in prokaryotic host cells.

The method according to the invention has advantages over methods known in the art- not only that it is a cheap production method due to the prokaryotic host cell used, surprisingly, a correctly folded molecule is secreted to the supernatant.

The skilled person can easily obtain the DNA sequence of a protein of interest to be expressed by the method according to the invention from suitable databases and clone it to be used in the method according to the invention.

Said signal peptide OmpA interacts with SecE and is delivered across the inner membrane by energy generated by SecA, which binds to Sec components (SecE-SecY). SecY forms a secretion pore to dispatch the recombinant DNA-derived protein according to the invention. The space between the outer membrane and inner membrane of Gram-negative bacteria, periplasm, has higher oxidative condition in comparison to the cytoplasmic space. This supports the formation of disulfide bonds and properly folding of the recombinant protein (e.g. K2S) in the periplasm to yield an active molecule. According to the present invention, the signal peptide will be cleaved off to produce a mature molecule. The complex of GspD secretin and GspS lipoprotein on the outer membrane serves as gate channel for secreting the recombinant protein according to the invention to the extracellular medium. This secretion process requires energy, which is generated in cytoplasm by GspE nucleotide-binding protein then transferred to the inner membrane protein (Gsp G-J, F and K-N). GspC transfers the energy to GspD by forming a cross-linker between a set of inner membrane protein (Gsp G-J, F and K-N) and GspD. Before crossing the outer membrane successfully, the recombinant protein is correctly folded.

Operably linked according to the invention means that the DNA encoding the heterologous protein (preferably comprising the nucleic acid encoding SEGN or SEGNSD at its N-terminal portion) is cloned in close proximity to the OmpA DNA into the vector in order to achieve expression of the OmpA-heterologous protein-fusion protein and to direct secretion outside the prokaryotic host cell. Typically, the majority of the heterologous protein is secreted and can then be purified by appropriate methods such as ammonium sulfate precipitation. The invention also includes the use of inducers such as IPTG or IPTG in

combination with glycerol, the improvement of the incubation condition and harvesting period to maximize the amount of active protein.

The inventors surprisingly found that the OmpA signal peptide alone or operatively linked to the amino acids characterized by the sequence SEGN or SEGNSD lead to secretion of the heterologous protein into the medium rather than accumulation in the periplasmatic space.

In a preferred embodiment, said DNA encoding the OmpA signal peptide may be fused to a short peptide characterized by the amino acid sequence SEGN or the coding nucleic acid sequence TCTGAGGGAAAC and located in the N-terminal portion or at the N-terminal portion of the heterologous protein. Thus, preferably, said fusion protein comprises OmpA-SEGN-heterologous protein. Even more preferred, said amino acids characterized by SEGN may be carry a point mutation or may be substituted by a non-natural amino acid. Even more preferred, there may be an amino acid or non-amino acid spacer between OmpA and SEGN or SEGN and the heterologous protein.

In a preferred embodiment, said DNA encoding the OmpA signal peptide may be fused to a short peptide characterized by the amino acid sequence SEGNSD or the coding nucleic acid sequence TCTGAGGGAAACAGTGAC and located in the N-terminal portion or at the N-terminal portion of the heterologous protein. Thus, preferably, said fusion protein comprises OmpA-SEGNSD-heterologous protein. Even more preferred, said amino acids characterized by SEGNSD may be carry a point mutation or may be substituted by a non-natural amino acid. Even more preferred, there may be an amino acid or non-amino acid spacer between OmpA and SEGNSD or SEGNSD and the heterologous protein.

Thus, in a preferred method according to the invention said the prokaryotic cell contains and expresses a vector comprising the DNA coding for said heterologous protein operably linked to the DNA coding for the signal peptide OmpA which is operably linked to the nucleic acid molecule defined by the sequence TCTGAGGGAAACAGTGAC or a functional derivative thereof.

Such heterologous proteins include, but are not limited to insulin, insulin-like growth factor, hGH, tPA, cytokines, e.g. interleukines (IL) such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, interferon (IFN) alpha, IFN beta, IFN gamma, IFN omega or IFN tau, tumor necrosisfactor (TNF)

TNF alpha and TNF beta, TRAIL; G-CSF, GM-CSF, M-CSF, MCP-1 and VEGF (I will **insert other possible proteins**).

The method according to the invention can be advantageously used for production of antibodies or fragments thereof. Such fragments include e.g. Fab fragments (Fragment antigen-binding = Fab). Fab fragments consist of the variable regions of both chains which are held together by the adjacent constant region. These may be formed by protease digestion, e.g. with papain, from conventional antibodies, but similar Fab fragments may also be produced in the mean time by genetic engineering. Further antibody fragments include F(ab')2 fragments, which may be prepared by proteolytic cleaving with pepsin. Using genetic engineering methods it is possible to produce shortened antibody fragments which consist only of the variable regions of the heavy (VH) and of the light chain (VL). These are referred to as Fv fragments (Fragment variable = fragment of the variable part). Since these Fv-fragments lack the covalent bonding of the two chains by the cysteines of the constant chains, the Fv fragments are often stabilised. It is advantageous to link the variable regions of the heavy and of the light chain by a short peptide fragment, e.g. of 10 to 30 amino acids, preferably 15 amino acids. In this way a single peptide strand is obtained consisting of VH and VL, linked by a peptide linker. An antibody protein of this kind is known as a single-chain-Fv (scFv). Examples of scFv-antibody proteins of this kind known from the prior art are described in Huston et al. (1988, PNAS 16: 5879-5883).

In recent years, various strategies have been developed for preparing scFv as a multimeric derivative. This is intended to lead, in particular, to recombinant antibodies with improved pharmacokinetic and biodistribution properties as well as with increased binding avidity. In order to achieve multimerisation of the scFv, scFv were prepared as fusion proteins with multimerisation domains. The multimerisation domains may be, e.g. the CH3 region of an IgG or *coiled coil* structure (helix structures) such as *Leucin-zipper* domains. However, there are also strategies in which the interaction between the VH/VL regions of the scFv are used for the multimerisation (e.g. dia-, tri- and pentabodies). By diabody the skilled person means a bivalent homodimeric scFv derivative (Hu et al., 1996, PNAS 16: 5879-5883). The shortening of the *Linker* in an scFv molecule to 5- 10 amino acids leads to the formation of homodimers in which an inter-chain VH/VL-superimposition takes place. Diabodies may additionally be stabilised by the incorporation of disulphide bridges. Examples of diabody-

antibody proteins from the prior art can be found in Perisic et al. (1994, Structure 2: 1217-1226).

By minibody the skilled person means a bivalent, homodimeric scFv derivative. It consists of a fusion protein which contains the CH3 region of an immunoglobulin, preferably IgG, most preferably IgG1 as the dimerisation region which is connected to the scFv via a *Hinge region* (e.g. also from IgG1) and a *Linker* region. The disulphide bridges in the *Hinge* region are mostly formed in higher cells and not in prokaryotes. Examples of minibody-antibody proteins from the prior art can be found in Hu et al. (1996, Cancer Res. 56: 3055-61).

By triabody the skilled person means a: trivalent homotrimeric scFv derivative (Kortt et al. 1997 Protein Engineering 10: 423-433). ScFv derivatives wherein VH-VL are fused directly without a linker sequence lead to the formation of trimers.

The skilled person will also be familiar with so-called miniantibodies which have a bi-, tri- or tetravalent structure and are derived from scFv. The multimerisation is carried out by di-, tri- or tetrameric coiled coil structures (Pack et al., 1993 Biotechnology 11:, 1271-1277; Lovejoy et al. 1993 Science 259: 1288-1293; Pack et al., 1995 J. Mol. Biol. 246: 28-34).

Therefore in another preferred method according to the invention an antibody or antibody fragment as described supra is produced.

The method according to the invention comprises prokaryotic host cells such as, but not limited to *Escherichia coli (E. coli)*, *Bacillus subtilis*, *Streptomyces*, *Pseudomonas*, *e.g. Pseudomonas putida*, *Proteus mirabilis* or *Staphylococcus*, e.g. *Staphylococcus carnosus*.

Preferably said host cells according to the invention are Gram-negative bacteria.

Preferably, a method according to the invention is also characterised in that the prokaryotic cell is *E. coli*. Suitable strains include, but are not limited to XL-1 blue, BL21(DE3), JM109, DH series, TOP10 and HB101.

Preferably, a method according to the invention is also characterised in that the following steps are carried out:

a) the DNA encoding the heterologous protein is amplified by PCR;

b) the PCR product is purified;

c) said PCR product is inserted into a vector comprising the DNA coding for OmpA signal peptide and the DNA coding for gpIII in such a way that said PCR product is operably linked upstream to the DNA coding for the OmpA signal sequence and linked downstream

to the DNA coding for gpIII of said vector;

d) that a stop codon is inserted between said heterologous protein and gpIII;

e) said vector is expressed by the prokaryotic cell

f) the heterologous protein is purified.

For step a) according to the invention the choice / design of the primers is important to clone the DNA in the right location and direction of the expression vector (see example 1). Thus, the primers as exemplified in example 1 and figure 4 comprise an important aspect of the present invention. With gp III of step c) gene protein III is meant which is present mainly in phagemid vectors. The stop codon is inserted to avoid transcription of gp III thus eventually leading to secretion of the heterologous protein of interest. Any suitable method for insertion of the stop codon may be employed such as site-directed mutagenesis (e.g. Weiner MP, Costa GL (1994) PCR Methods Appl 4(3):S131-136; Weiner MP, Costa GL, Schoettlin W, Cline J, Mathur E, Bauer JC (1994) Gene 151(1-2):119-123; see also example 1).

Any vector may be used in the method according to the invention, preferably said vector is a phagemid vector (see below).

The untranslated region may contain a regulatory element, such as e.g. a transcription initiation unit (promoter) or enhancer. Said promoter may, for example, be a constitutive, inducible or development-controlled promoter. Preferably, without ruling out other known promoters, the constitutive promoters of the human Cytomegalovirus (CMV) and Rous sarcoma virus (RSV), as well as the Simian virus 40 (SV40) and Herpes simplex promoter. Inducible promoters according to the invention comprise antibiotic-resistant promoters, heat-shock promoters, hormone-inducible „Mammary tumour virus promoter" and the metallothioneine promoter. Preferred promotors include T3 promotor, T7 promotor, Lac/ara1 and Ltet0-1.

More preferably, a method according to the invention is also characterised in that the DNA encoding the heterologous protein is preceeded by a lac promotor and/or a ribosomal binding site such as the Shine-Dalgarno sequence (see also example).

Suitable vectors according to the invention include, but are not limited to viral vectors such as e.g. Vaccinia, Semliki-Forest-Virus and Adenovirus, phagemid vectors and the like. Preferred are vectors which can be advantageously used in E. coli, but also in any other

prokaryotic host such as pPROTet.E, pPROLar.A, members of the pBAD family, pSE family, pQE family and pCAL.

Another preferred embodiment of the invention relates to the vector pComb3HSS containing a DNA according to the invention, wherein the expression of the gp III protein is suppressed or inhibited by deleting the DNA molecule encoding said gp III protein or by a stop codon between the gene coding for a a polypeptide containing the heterologous protein and the protein III gene.

Preferably, a method according to the invention is also characterised in that the heterologous protein is selected from human tissue plasminogen activator (tPA) or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant therof. Such fragments, allelic variants, functional variants, variants based on the degenerative nucleic acid code, fusion proteins with an tPA protein according to the invention, chemical derivatives or a glycosylation variant of the tPA proteins according to the invention may include one, several or all of the following domains or subunits or variants thereof:

1. Finger domain (4-50)
2. Growth factor domain (50-87)
3. Kringle 1 domain (87-176)
4. Kringle 2 domain (176-262)
5. Protease domain (276-527)

The numbering/naming of the domains is according to Genbank accession number GI 137119 or Nature 301 (5897), 214-221 (1983).

More preferably, a method according to the invention is also characterised in that the heterologous protein is selected from the Kringle 2 (4.) plus Serine protease (5.) K2S variant of human tissue plasminogen activator or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant therof.

More preferably, a method according to the invention is also characterised in that the vector is a phagemid vector comprising the DNA coding for OmpA signal peptide and the DNA coding for gpIII.

The following example is intended to aid the understanding of the invention and should in no way be regarded as limiting the scope of the invention.

Example 1

MATERIALS AND METHODS

Primer design. In order to amplify a specific part of tPA gene, a pair of primers SK2/174 [5′ GAGGAGGAGGTGGCCCAGGCGGCCTCTGAGGGAAACAGTGAC 3′] and ASSP [5′ GAGGAGGAGCTGGCCGGCCTGGCCCGGTCGCATGTTGTCACG 3′] were synthesized (Life Technologies, Grand Island, NY). These primers were designed based on the human tPA gene retrieved from NCBI databases (g137119). They were synthesized with Sfi I end cloning sites (underlined) in such a way that the reading frame from the ATG of the gpIII gene in phagemid vector, pComb3HSS, will be maintained throughout the inserted sequence.

Another primer set for site-directed mutagenesis was designed to anneal at the sequence situated between K2S gene and gene III in pComb3H-K2S. The sequence of primers with mutation bases (underlined) for generating a new stop codon were MSTPA [5′ ACATGCGACCGTGACAGGCCGGCCAG 3′] and MASTPA [5′ CTGGCCGGCCTGTCACGGTCGCATGT 3′].

Amplification of K2S gene by PCR. One μg SK2/174 and ASSP primers together with 50 ng of p51-3 template (obtained from Dr. Hiroshi Sasaki, Fujisawa Pharmaceutical, Japan) were suspended in 100 μl PCR mixture. An amount of 2.5 U Taq polymerase (Roche Molecular Biochemicals, Indianapolis, IN) was finally added to the solution. The titrated amplification condition was initiated with jump start at 85°C for 4 min, then denaturation at 95°C for 50 sec, annealing at 42°C for 50 sec, extension at 72°C for 1.5 min. Thirty five rounds were repeatedly performed. The mixture was further incubated at 72°C for 10 min. The amplified product of 1110 bp was subsequently purified by QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). The correctness of purified product was confirmed by restriction enzymes.

Construction of phagemid expressing K2S. The purified PCR product of K2S and pComb3HSS phagemid (kindly provided by Dr. Carlos F. Barbas, Scripps Institute, USA) were digested with Sfi I (Roche Molecular Biochemicals, Indianapolis, IN) to prepare specific cohesive cloning sites. Four μg of the purified PCR product was digested with 60 U

of Sfi I at 50°C for 18 h. For pComb3HSS, 20 μg of phagemid vectors were treated with 100 U of Sfi I. Digested products of purified PCR product of K2S and pComb3HSS (~3300 bp) were subsequently gel-purified by the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). T4 ligase (Roche Molecular Biochemicals, Indianapolis, IN) of 5 U were introduced to the mixture of 0.7 μg of purified Sfi I-digested pComb3HSS and 0.9 μg of purified Sfi I-digested PCR product. Ligation reaction was incubated at 30°C for 18 h. The newly constructed phagemid was named pComb3H-K2S.

Transformation of XL-1 Blue. Two hundred μl of $CaCl_2$ competent E. coli XL-1 Blue (Stratagene, La Jolla, CA) were transformed with 70 ng of ligated or mutated product. The transformed cells were propagated by spreading on LB agar containing 100 μg/ml ampicillin and 10 μg/ml tetracycline (Sigma, Saint Louis, MO). After cultivation at 37°C for 18 h several antibiotic resistant colonies were selected for plasmid minipreps by using the alkaline lysis method. Each purified plasmid was subjected to Sfi I restriction site analysis. A transformant harboring plasmid with the correct Sfi I restriction site(s) was subsequently propagated for 18 h at 37°C in 100 ml LB broth with ampicillin 100 μg/ml and tetracycline 10 μg/ml. A plasmid maxiprep was performed using the QIAGEN Plasmid Maxi Kit (QIAGEN, Hilden, Germany). The purified plasmid was reexamined for specific restriction sites by Sfi I and sequenced by AmpliTaq DNA Polymerase Terminator Cycle Sequencing Kit (The Perkin-Elmer Corporation, Forster City, CA).

Site-directed mutagenesis of pComb3H-K2S. 10 ng of pComb3H-K2S template were mixed with 125 ng of MSTPA and MASTPA primers. PfuTurbo DNA polymerase (Stratagene, LA Jolla, CA) of 2.5 U was added to the mixture for cycle amplification. The reaction started with one round of 95°C for 30 sec. Then it was followed by 16 rounds consisting of 95°C for 30 sec, 55°C for 1 min, and 68°C for 9 min. The reaction tube was subsequently placed on ice for 2 min. In order to destroy the template strands, 10 U of Dpn I restriction enzyme (Stratagene, LA Jolla, CA) were added to the amplification reaction and incubated for 1 h at 37°C. This synthesized product (MpComb3H-K2S) was further used to transform E. coli XL-1 Blue.

Preparation of phage-display recombinant-K2S. After pComb3H-K2S was transformed to XL-1 Blue, the phage display technique was performed. A clone of pComb3H-K2S transformed XL-1 Blue was propagated in 10 ml super broth containing ampicillin 100 μg/ml and tetracycline 10 μg/ml at 37°C until the O.D. [600 nm] of 1.5 was reached. The bacterial culture was subsequently propagated in 100 ml of the same medium and culture for 2 h. An amount of 10^{12} pfu of VCSM13 helper phage (Stratagene, La Jolla, CA) was used to infect the transformed XL-1 Blue. After 3 h incubation, kanamycin at a final concentration of 70 μg/ml final concentration was added to culture. The culture was left shaking (200 RPM) for 18 h at 37°C. Bacteriophages which harbored K2S on gp3 (K2S-ϕ) were then harvested by adding 4% w/v PEG MW 8000 (Sigma, Saint Louis, MO) and 3% w/v NaCl. Finally, the harvested phage was resuspended in 2 ml PBS pH 7.4. The phage number was determined by infecting XL-1 Blue. The colony-forming unit per milliliter (cfu/ml) was calculated as described previously (21).

Expression of recombinant-K2S in shaker flasks. MpComb3H-K2S transformed XL-1 Blue was cultivated in 100 ml super broth (3% w/v tryptone, 2% w/v yeast extract and 1% w/v MOPS) at pH 7.0 in the presence of ampicillin (100 μg/ml) at 37°C until an O.D. [600 nm] of 0.8 was reached. Subsequently, the protein synthesis was induced by 1 mM of IPTG (Promega, Madison, WI). The bacteria were further cultured shaking (200 RPM) for 6 h at 30°C. The culture supernatant was collected and precipitated with 55% saturated ammonium sulfate (32). The precipitate was reconstituted with PBS, pH 7.2, and dialysed in the same buffer solution at 4°C for 18 h. Periplasmic proteins from bacterial cells were extracted by using a chloroform shock as previously described by Ames et al. (2).

Immunoassay quantification of recombinant-K2S. In order to detect r-K2S, solid phase was coated with monoclonal anti-kringle 2 domain (16/B) (generously provided by Dr. Ute Zacharias, Central Institute of Molecular Biology, Berlin-Buch, Germany). The standard ELISA washing and blocking processes were preformed. Fifty μl of 10^{11} cfu/ml of K2S-ϕ or secretory r-K2S were added into each anti-kringle 2 coated well. Antigen-antibody detection was carried out as follows. Either sheep anti-M13 conjugated HRP (Pharmacia Biotech, Uppsala, Sweden) or sheep anti-tPA conjugated HRP (Cedarlane, Ontario, Canada), was added to each reaction well after the washing step. The substrate TMB was

subjected to every well and the reaction was finally ceased with H_2SO_4 solution after 30 min incubation. The standard melanoma tPA 86/670 (National Institute for Biological Standards and Control, Hertfordshine, UK) was used as positive control.

Amidolytic activity assay. A test kit for the detection of tPA amidolytic activity was purchased from Chromogenix (Molndal, Sweden). The substrate mixture containing plasminogen and S-2251 was used to determine serine protease enzymatic activity. The dilution of 10^{-2} of each ammonium precipitated sample was assayed with and without stimulator, human fibrinogen fragments. The assay procedure was according to the COASET t-PA manual.

SDS-PAGE and immunoblotting. The dialysed precipitate-product from culture supernatant was further concentrated 10 folds with centricon 10 (AMICON , Beverly, MA). The concentrated sample was subjected to protein separation by SDS-PAGE, 15% resolving gel, in the reducing buffer followed by electroblotting to nitrocellulose. The nitrocellulose was then blocked with 4% skimmed milk for 2 hr. In order to detect r-K2S, a proper dilution of sheep anti-tPA conjugated HRP was applied to the nitrocellulose. The immunoreactive band was visualized by a sensitive detection system, Amplified Opti-4CN kit (BIORAD, Hercules, CA).

Copolymerized plasminogen polyacrylamide gel electrophoresis. An 11% resolving polyacrylamide gel was copolymerized with plasminogen and gelatin as previously described by Heussen et al. (14). The stacking gel was prepared as 4 % concentration without plasminogen and gelatin. Electrophoresis was performed at 4°C at a constant current of 8 mA. The residual SDS in gel slab was removed after gentle shaking at room temperature for 1h in 2.5% Triton X-100. Then the gel slab was incubated in 0.1 M glycine-NaOH, pH 8.3, for 5 h at 37°C. Finally, the gel slab was stained and destained by standard Coomassie brilliant blue (R-250) dying system. The location of the peptide harboring enzymatic activity was not stained by dye in contrast to blue-paint background.

RESULTS

Construction of K2S gene carrying vector. From the vector p51-3 we amplified the kringle 2 plus ther serine protease portion of tPA (Ser^{174} in kringle 2 domain to Pro^{527} in the

serine protease) using primers SK2/174 and ASSP. The amplified 1110 bp product was demonstrated by agarose gel electrophoresis (Fig. 1, lane 2) and was inserted into pComb3HSS phagemid by double Sfi I cleavage sites on 5′ and 3′ ends in the correct reading frame. Thus a new vector, pComb3H-K2S, harboring the K2S was generated. In this vector K2S is flanked upstream by the OmpA signal sequence and donwstream by gp3. The correct insertion of K2S was verified both by restriction analysis with Sfi I (Fig. 2, lane 3), PCR-anaysis (demonstration of a single band at 1110 bp), and DNA sequencing. The schematic diagram of pComb3H-K2S map is given in Fig. 3.

Phage-displayed r-K2S. VCSM13 filamentous phage was used to infect pComb3H-K2S transformed XL-1 Blue, X[K2S]. VCSM13 was propagated and incorporated the K2S-gp3 fusion protein during the viral packaging processes. The harvested recombinant phage (K2S-ϕ) gave a concentration of 5.4×10^{11} cfu/ml determined by reinfecting XL-1 Blue with PEG-precipitated phages. These recombinant phage particles were verified for the expression of r-K2S by sandwich ELISA. The phage-bound heterologous K2S protein was recognized by the monoclonal anti-kringle 2 antibody (16/B) by using sheep anti-tPA conjugated HRP antibody detection system. The absorbance of this assay was 1.12 ± 0.03 (Table 1). The amount of K2S detectable on 10^{12} phage particles is equal to 336 ng of protein in relation to the standard melanoma tPA. In order to corroborate that K2S-gp3 fusion protein was associated with phage particles, sheep anti-tPA conjugated HRP antibody was substituted by sheep anti-M13 antibody conjugated HRP. This immuno-reaction exhibited an absorbance of 1.89 ± 0.07 (Table 1). In contrast, if the capture antibody was sheep anti-M13 antibody, extremely low K2S was observed with sheep anti-tPA antibody conjugated HRP; the absorbance was only 0.17 ± 0.01 (Table 1). This suggested that only a minority of purified phage particles carried K2S-gp3 fusion protein. VCSM13 prepared from non-transformed XL-1 Blue was used as a negative control.

Construction of MpComb3H-K2S. We generated a stop codon between K2S and gp3 in pComb3H-K2S with the aid of the mutagenic primers (MSTPA and MASTPA) (Fig. 4). In order to enrich the newly synthesized and mutated MpComb3H-K2S, the cycle amplification mixture was thoroughly digested with Dpn I to degrade the old dam methylated pComb3H-K2S template (Dpn I prefers dam methylated DNA). After

transforming of XL-1 Blue with MpComb3H-K2S, a transformant XM[K2S] was selected for further study. As a consequence of bp substitution, one Sfi I cleavage site close to the 3' end of K2S gene was lost after site-directed mutagenesis. A linear version of Sfi I cleaved MpComb3H-K2S was observed at 4319 bp without the appearance of inserted K2S gene fragment (Fig. 5, lane 3). Thus, the K2S gene encoding by MpComb3H-K2S was expressed in non-gp3 fusion form in XM[K2S].

Expression and purification of K2S. K2S expression in XM[K2S] was induced by IPTG. r-K2S was detectable by using ELISA both in the periplasmic space and in the culture supernatant . The amount of the heterologous protein in each preparation was determined by sandwich ELISA and related to the standard tPA. From 100 ml of the bacterial culture in shaker flask with the O.D. [600 nm] of 50, the periplasmic fraction yielded 1.38 μg of r-K2S (approximately 32%) whereas 2.96 μg of r-K2S (approximately 68%) was obtained in the ammonium precipitated culture supernatant. Sandwich ELISA was used to verify the PEG precipitated phage from VCSM13 infected XM[K2S]. No r-K2S captured by monoclonal anti-kringle 2 antibody was detected by anti-M13 conjugated HRP, indicating that K2S is not presented on the phage particles if gp3 is missing.

Amidolytic activity measurement. If serine protease domain is present in the sample, plasminogen will be converted to plasmin. The produced plasmin will further digest the S-2251 substrate to a colour product, p-nitroaniline, which has a maximum absorbance at 405 nm. The specific activity of the recombinant product is in accord with the absorbance. The fibrinogen-dependent enzymatic activity of each sample i.e. K2S-ϕ, periplasmic r-K2S or culture supernatant r-K2S, was evaluated and compared. Both K2S-ϕ and periplasmic r-K2S illustrated notably low enzymatic activity, which was below the sensitivity of the test (0.25 IU/ml). The culture supernatant r-K2S gave the fibrinogen-dependent enzymatic activity of 7 IU/ml. Thus, from 100 ml culture we obtained a total of 700 IU enzymatic activity. Without fibrinogen no enzymatic activity of the r-K2S purified from culture supernatant was observed - whereas standard melanoma tPA showed some activity.

Demonstration of recombinant protein by immunoblotting. Partially purified K2S from culture supernatant of XM[K2S] revealed a molecular mass of 39 kDa by using sheep anti-

tPA antibodies (Fig. 6). The negative control, partially purified culture supernatant of non-transformed XL1-Blue, contained no reactive band with a similar size.

Localization of active enzyme by PAGE. The plasminogen has been copolymerized and immobilized with gelatin in the polyacrylamide gel prior to electrophoresis. The ammonium sulfate precipitated culture supernatants of XL-1 Blue, XL-1 Blue transformed with pComb3HSS and XM[K2S] were analyzed (Fig. 7). All samples were processed in non-reducing condition to preserve the correct conformation and activity of the serine protease domain. Transparent areas of serine protease digested plasminogen were observed only in the ammonium sulfate precipitated culture supernatants of XM[K2S] at 34 and 37 kDa postions. The other samples gave no clearing zones. The positive control lane of standard melanoma tPA also demonstrated enzymatic activity at 66 and 72 kDa positions.

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FIGURE LEGENDS

FIG. 1. Validation of PCR amplification product of the K2S gene from the p51-3 vector by using SK2/174 and ASSP primers. Lane 1 shows 1 kb marker (Roche Molecular Biochemicals, Indianapolis, IN). Lane 2 was loaded with 1 μl of amplified product. A single band at 1110 bp is depicted. The electrophoresis was performed on a 1% agarose gel.

FIG. 2. Identification of inserted K2S gene at 1110 bp (*) after Sfi I digested pComb3H-K2S was demonstrated in lane 3. Lane 1 shows 1 kb marker. Lane 2 was loaded with uncut pComb3H-K2S. The electrophoresis was performed on a 1% agarose gel.

FIG. 3. Scheme of pComb3H-K2S showing two Sfi I cloning sites into which the K2S gene was inserted. Signal sequence (OmpA), ribosome binding site (RIBS), lac promotor, and gpIII gene are also depicted.

FIG. 4. Schematic diagram of the mutation site at the junction between the K2S and gpIII genes on pComb3H-K2S. The annealing site of pComb3H-K2S is bound with a set of mutation primers (MSTPA and MASTPA) containing modified oligonucleosides (underlined). After performing the cycle amplification, the Sfi I site 1 (in bold) is modified and lost in the newly synthesized strand.

FIG. 5. Characterization of newly synthesized MpComb3H-K2S by the Sfi I restriction enzyme. A single band at 4319 bp that refers to a single cleavage site of MpComb3H-K2S is observed in lane 3. No inserted K2S band at 1110 bp can be visualized. Lane 1 shows 1 kb marker. Lane 2 was loaded with uncut MpComb3H-K2S. The electrophoresis was performed on a 1% agarose gel.

FIG. 6. Identification of immunological reactive band with of recombinant protein purified from XM[K2S] culture supernatant with sheep anti-tPA conjugated HRP. Lane 1 was loaded with 40 ng of standard melanoma tPA (86/670), which showed the reactive band at

70 kDa. The partially purified and concentrated culture supernatants from non-transformed XL1- Blue and XM[K2S] were applied to lane 2 and 3 respectively. The distinct reactive band was particularly demonstrated in lane 3 at 39 kDa.

FIG. 7. Molecular weight determination of extracellular r-K2S harboring active serine protease domain by copolymerized plasminogen polyacrylamide gel electrophoresis. Lane 1 contained the indicated molecular weight standards ($\times 10^{-3}$), SDS-6H (Sigma, Saint Louis, MO). Fifty μg of the 55% saturated ammonium sulfate precipitated culture supernatant of XL-1 Blue, Xl-1 Blue transformed with pComb3HSS, and XM[K2S] were loaded in lane 2, 3, and 4 respectively. Lane 5 contained 50 mIU of standard melanoma tPA (86/670). Transparent zones of digested plasminogen in polyacrylamide gel are visible only in lane 4 at molecular weight of 34 and 37 kDa (B) and lane 5 at molecular weight of 66 and 72 kDa (A).

FIG. 8. Structure A

Native K2S molecule from amino acids 174-527 without modification.

FIG. 9. Structure B-0

Native K2S molecule from amino acids 197-527 without modification.

FIG. 10. Structure B-1

K2S molecule from amino acids 193-527, wherein to Structure B-0 of Fig. 9 the amino acids SEGN were added at the N-terminal portion.

FIG. 11. Structure B-2

K2S molecule from amino acids 193-527, as in Fig. 10, wherein Cys-261 was exchanged for Ser.

FIG. 12. Structure B-3

K2S molecule from amino acids 191-527, wherein to Structure B-0 of Fig. 9 the amino acids SEGNSD were added at the N-terminal portion.

FIG. 13. Structure B-4

K2S molecule from amino acids 191-527, as in Fig. 12, wherein Cys-261 was exchanged for Ser.

FIG. 14. Structure C

Native K2S molecule from amino acids 220-527 without modification. This molecule may be further modified in a similar manner as disclosed for structure B in figures 10-13.

FIG. 15. Structure D

Native K2S molecule from amino acids 260-527 without modification. This molecule may be further modified in a similar manner as disclosed for structure B in figures 10-13.

FIG. 16. tPA molecule

TABLE 1. Detection of r-K2S molecule in phage preparation by sandwich ELISA

Capture antibody	Tracer antibody (conjugated HRP)			
	Anti-tPA		Anti-M13	
	K2S-ϕ	VCSM13[a]	K2S-ϕ	VCSM13
Anti-kringle 2[b]	1.12 ± 0.04[c]	0.12 ± 0.03	1.89 ± 0.02	0.16 ± 0.02
Anti-M13	0.17 ± 0.01	0.14 ± 0.05	1.91 ± 0.02	1.88 ± 0.03

[a] VCSM13 was harvested from XL-1 Blue transformed with pComb3HSS.

[b] Mouse monoclonal anti-kringle 2 (16/B) was used. The other antibodies were prepared from sheep immunoglobulin.

[c] Value is mean of absorbance of each sample which was assayed in triplicate.

Claims

1. Method for the production of recombinant DNA-derived heterologous protein in prokaryotic cells, wherein said heterologous protein is secreted extracellularly as an active and correctly folded protein, characterized in that the prokaryotic cell contains and expresses a vector comprising the DNA coding for said heterologous protein operably linked to the DNA coding for the signal peptide OmpA or a functional derivative thereof.

2. Method according to claim 1, characterised in that said the prokaryotic cell contains and expresses a vector comprising the DNA coding for said heterologous protein operably linked to the DNA coding for the signal peptide OmpA which is operably linked to the nucleic acid molecule defined by the sequence TCTGAGGGAAACAGTGAC or a functional derivative thereof.

3. Method according to claim 1 or 2, characterised in that the prokaryotic cell is E. coli.

4. Method according to one of claims 1 to 3, characterised in that the the following steps are carried out:

a) the DNA encoding the heterologous protein is amplified by PCR;

b) the PCR product is purified;

c) said PCR product is inserted into a vector comprising the DNA coding for OmpA signal peptide and the DNA coding for gpIII in such a way that said PCR product is operably linked upstream to the DNA coding for the OmpA signal sequence and linked downstream to the DNA coding for gpIII of said vector;

d) that a stop codon is inserted between said heterologous protein and gpIII;

e) said vector is expressed by the prokaryotic cell;

f) the heterologous protein is purified.

5. Method according to one of claims 1 to 4, characterised in that the heterologous protein is selected from human tissue plasminogen activator or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant therof.

6. Method according to one of claims 1 to 5, characterised in that the heterologous protein is selected from the K2S variant of human tissue plasminogen activator or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant therof.

7. Method according to one of claims 1 to 6, characterised in that the vector is a phagemid vector comprising the DNA coding for OmpA signal peptide and the DNA coding for gpIII.

8. Method according to one of claims 1 to 7, characterised in that the vector is the pComb3HSS phagemid.

9. Method according to one of claims 1 to 8, characterised in that the DNA Sequence of OmpA comprises the following sequence:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTG GCCCAGGCGGCC

10. Method according to one of claims 1 to 9, characterised in that the DNA Sequence of OmpA consists of the following sequence:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTG GCCCAGGCGGCC

11. Method according to one of claims 1 to 10, characterised in that the DNA of the heterologous protein is preceeded by a lac promotor and/or a ribosomal binding site.

Abstract

The invention belongs to the field of protein production in prokaryotic cells.

The invention relates to methods for the production of recombinant DNA-derived heterologous protein in prokaryotic cells, wherein said heterologous protein is secreted extracellularly as an active and correctly folded protein, and the prokaryotic cell contains and expresses a vector comprising the DNA coding for said heterologous protein operably linked to the DNA coding for the signal peptide OmpA.

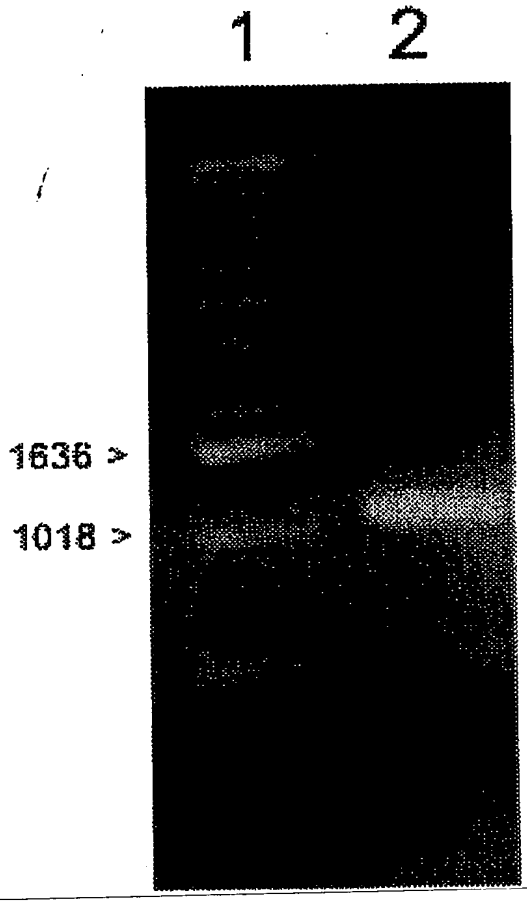


Fig 1

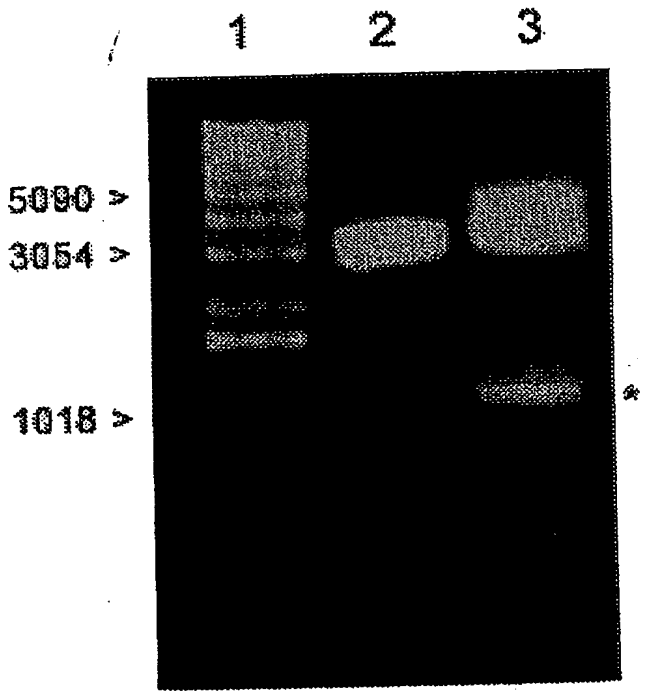
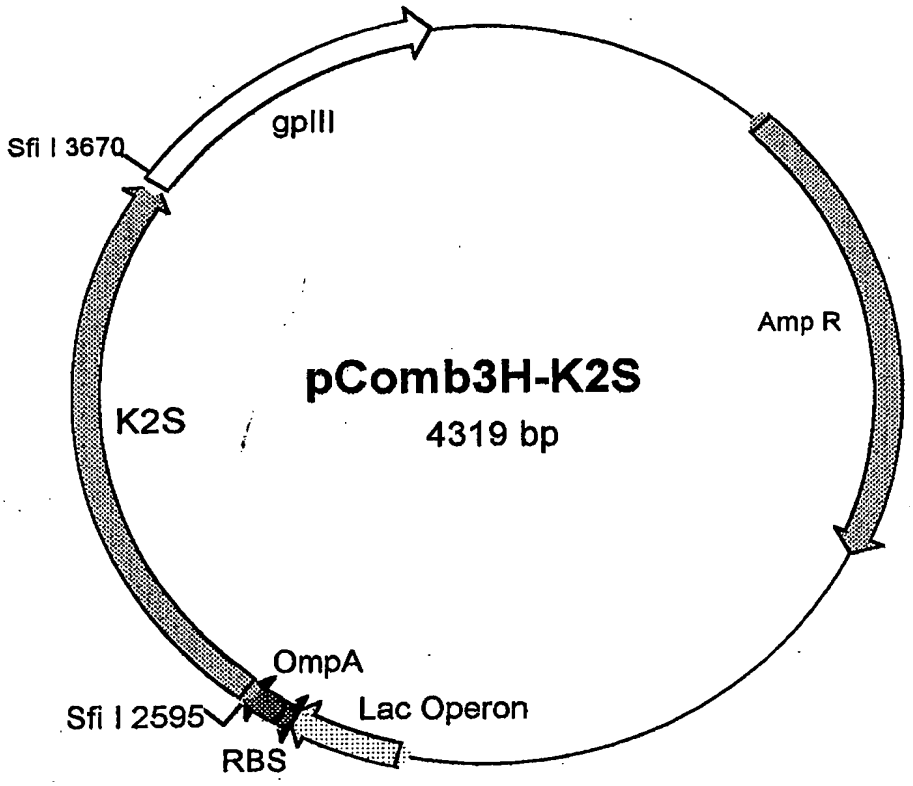


Fig 2



Lac operon	2316-2513
RBS	2514-2533
Omp A	2534-2599
K2S	2600-3664
gp III	3665-4219
Amp R	515- 1394

Figure 3

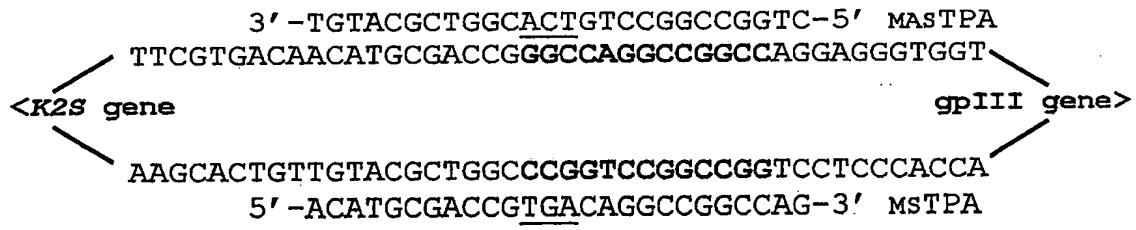


Figure 4

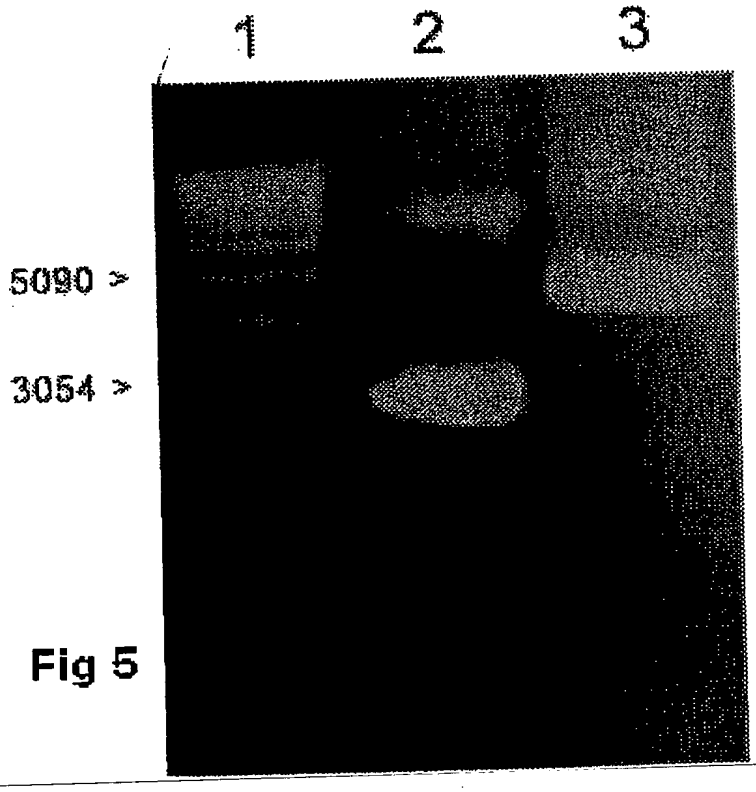


Fig 5

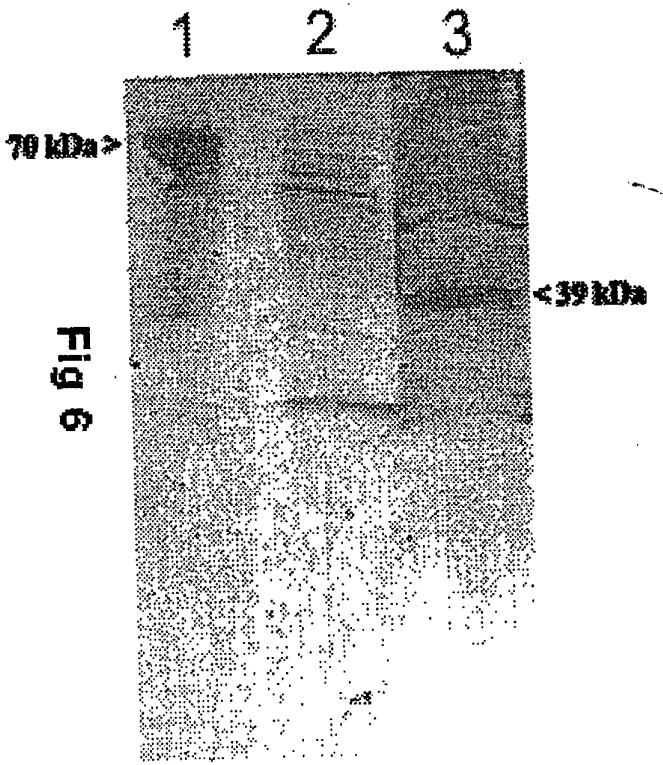


Fig 6

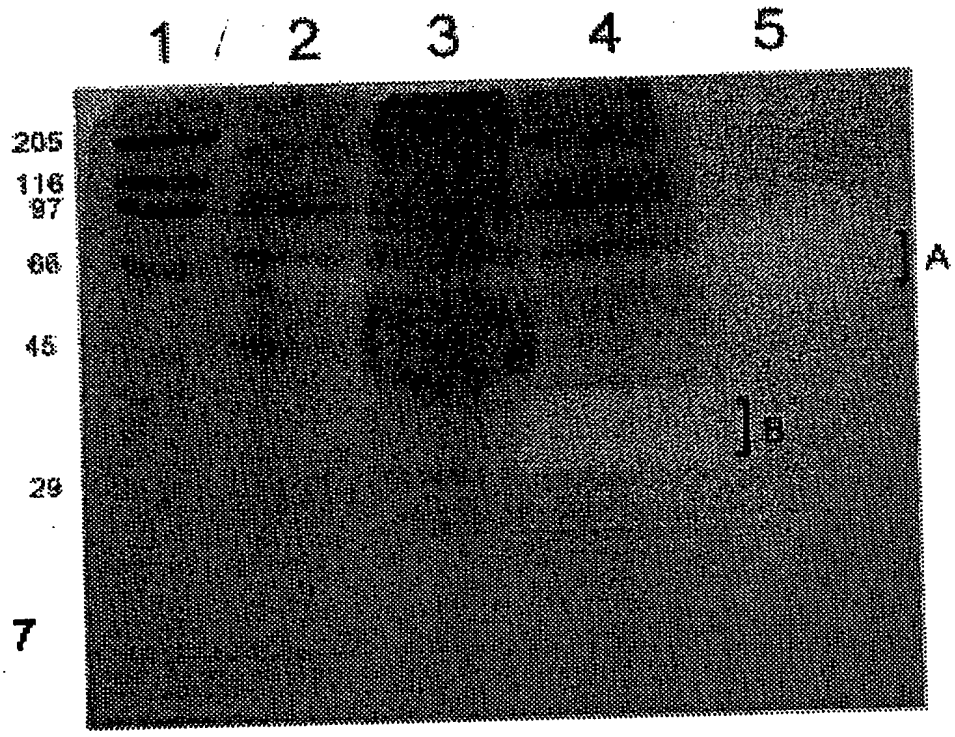


Fig 7

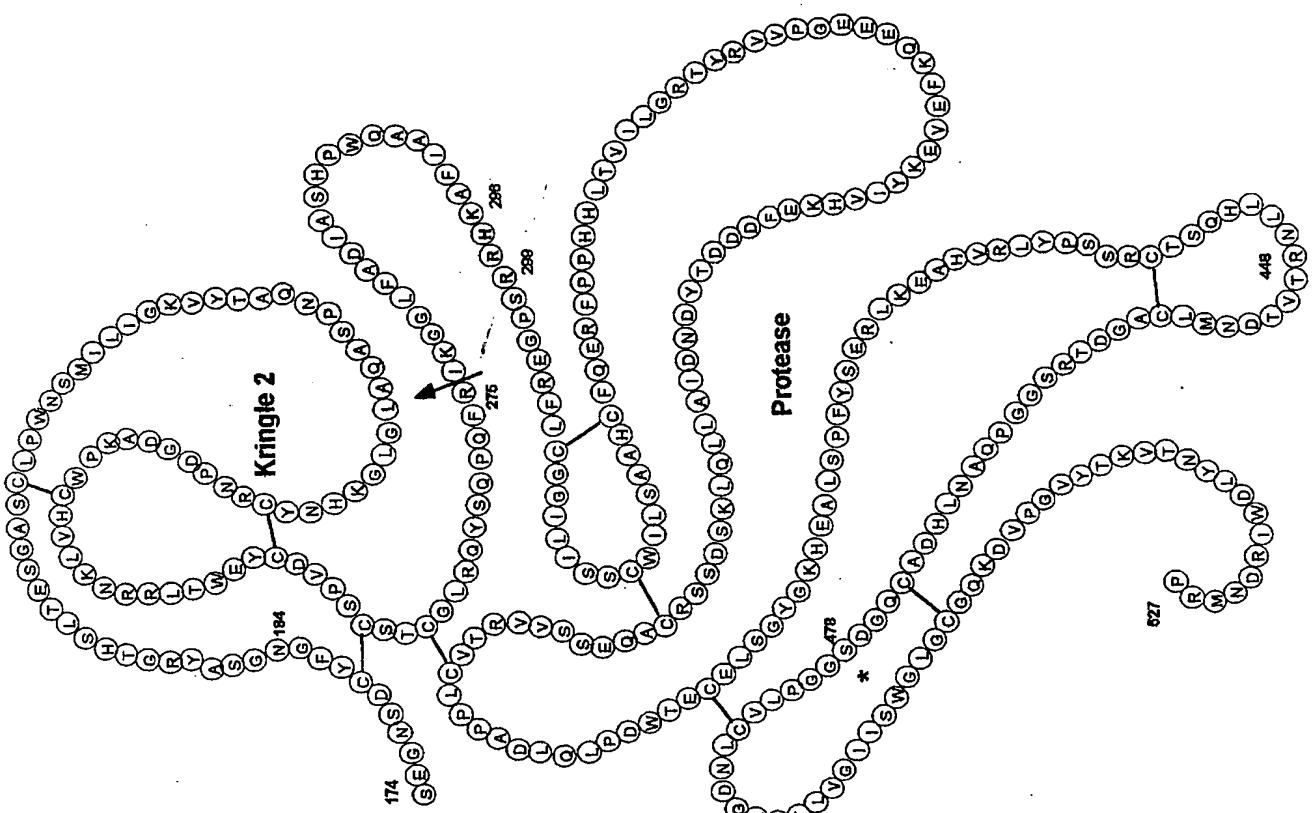


Fig. 8

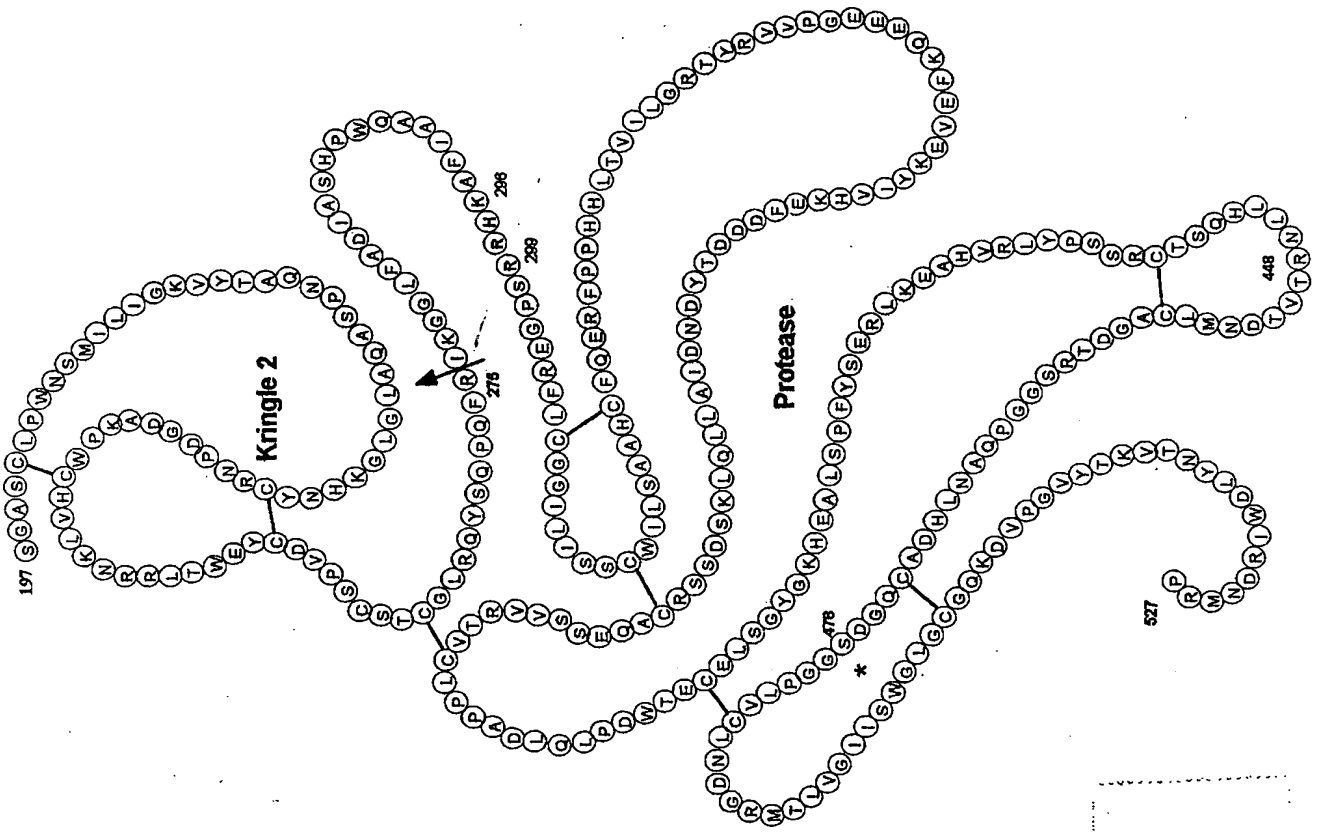


Fig. 9

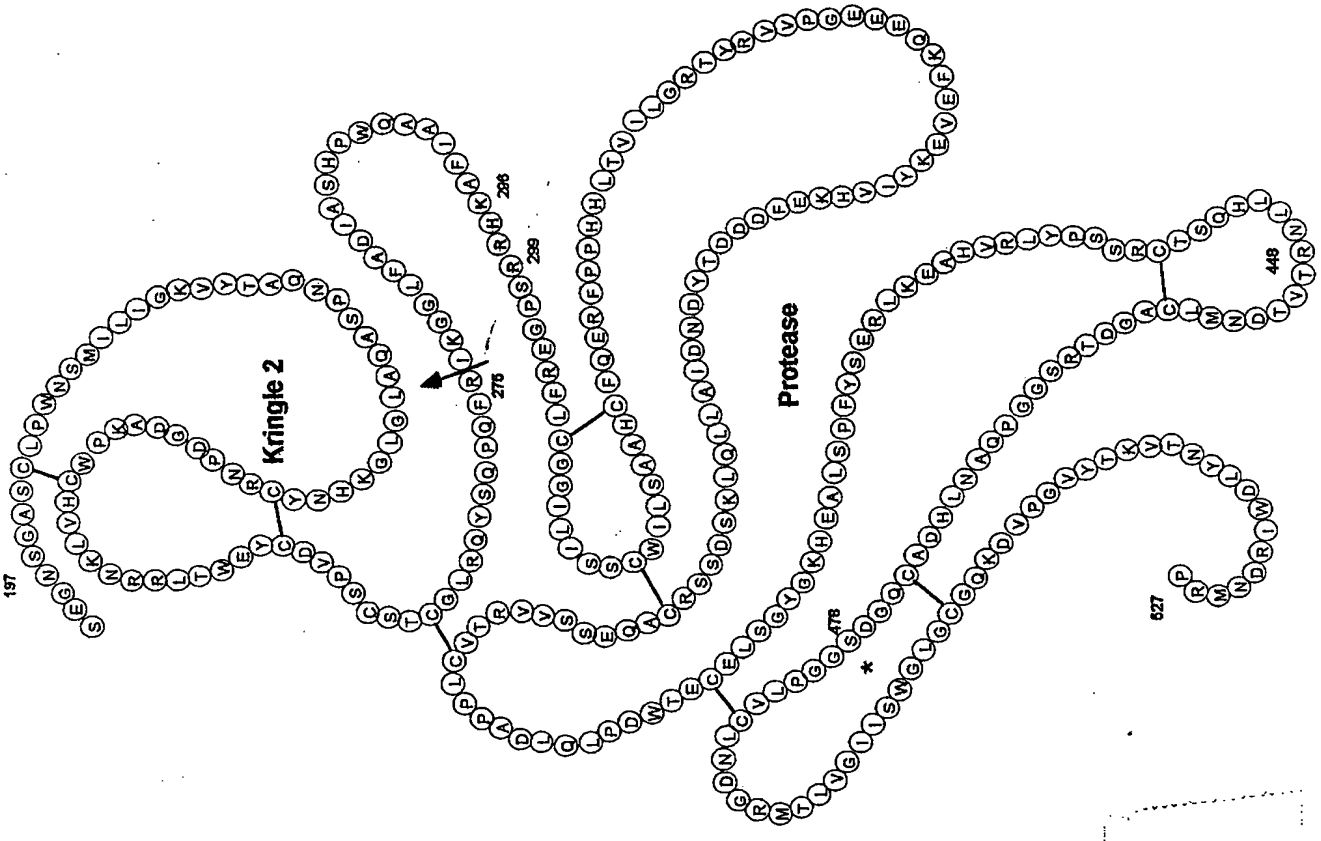


Fig. 10

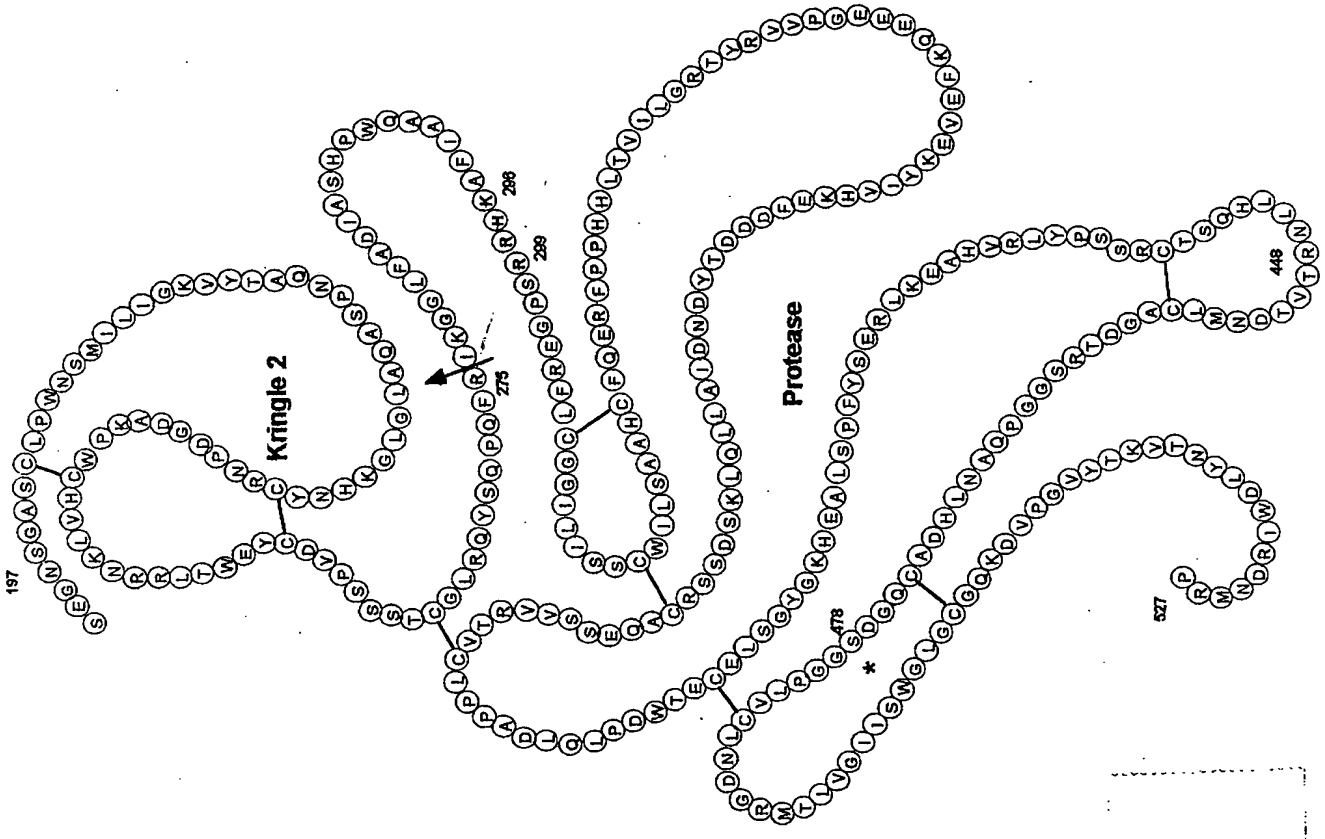


Fig. 11

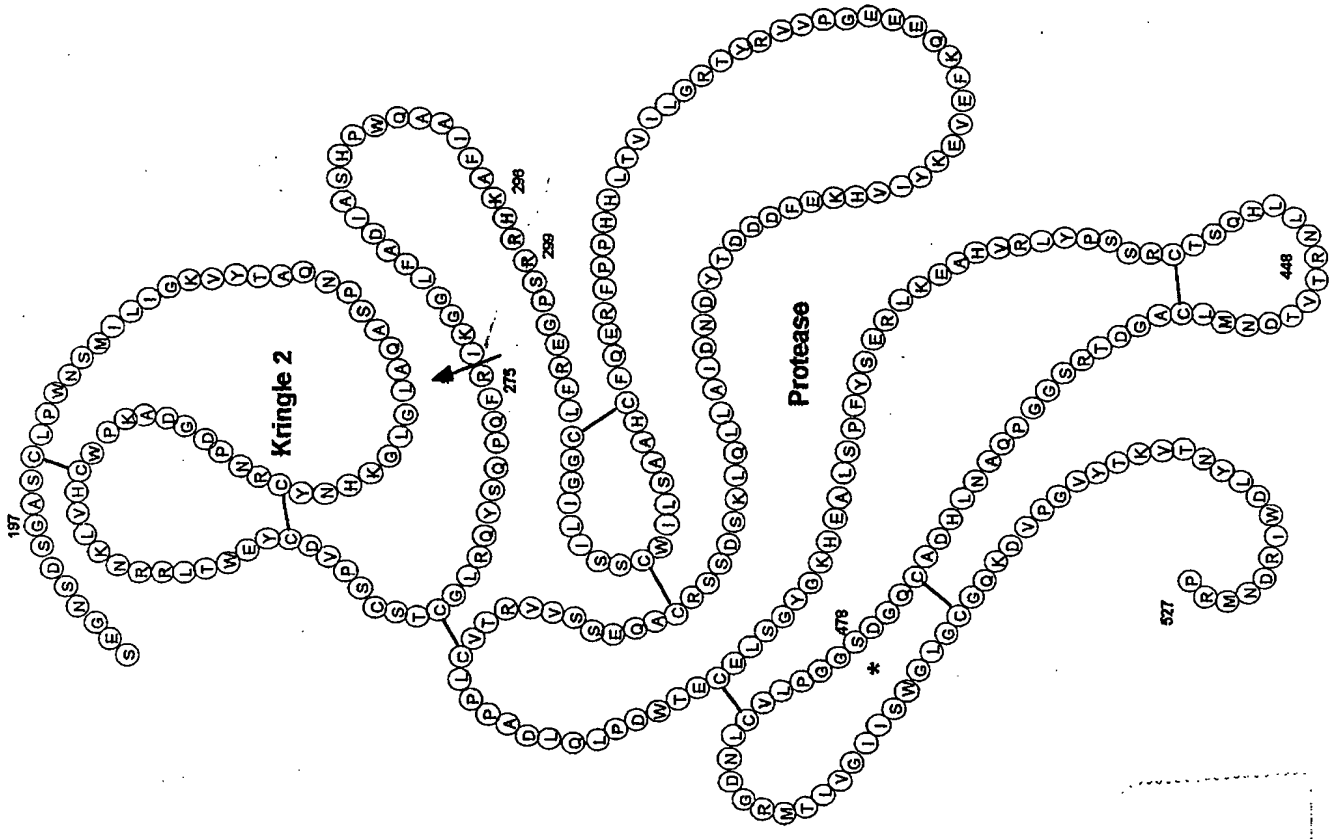


Fig. 12

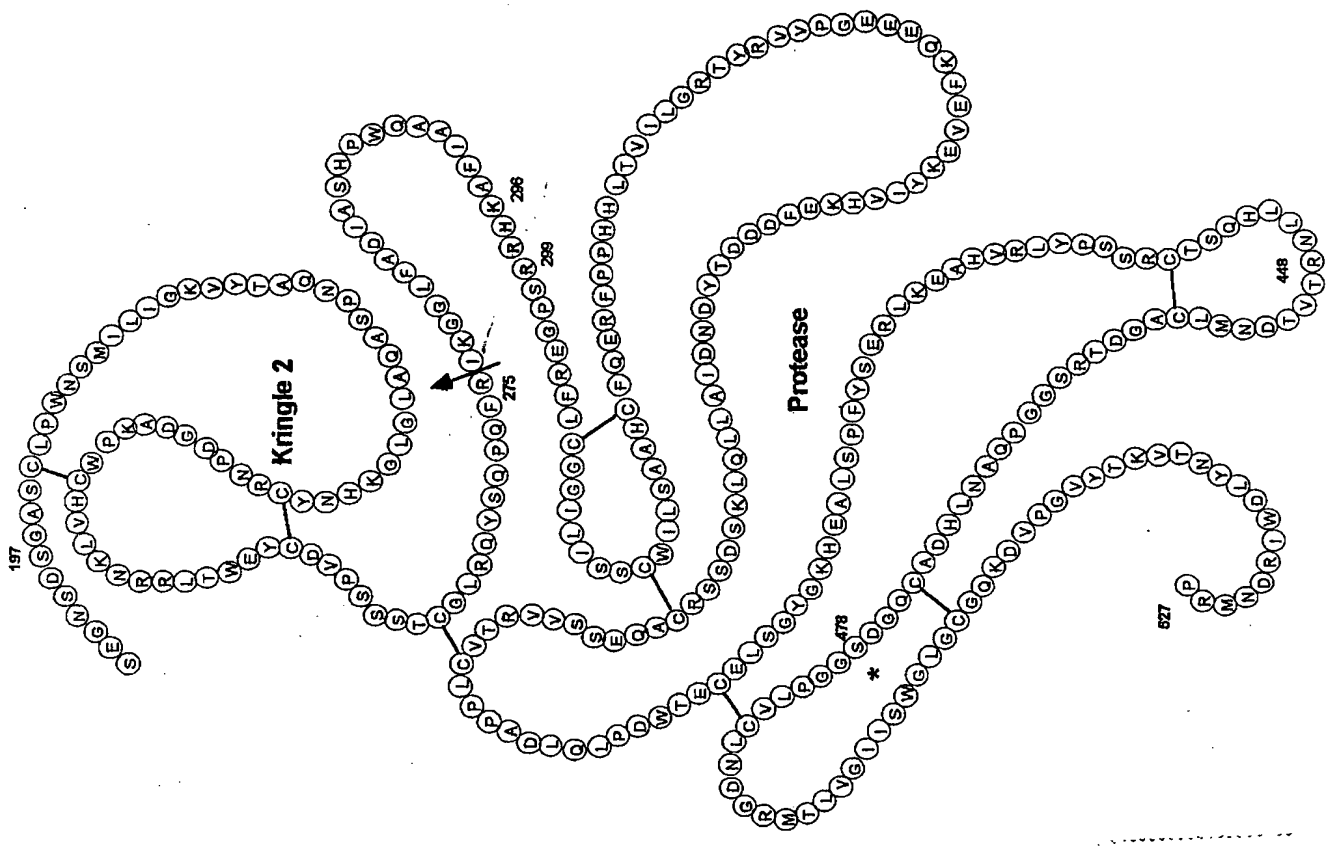


Fig. 13

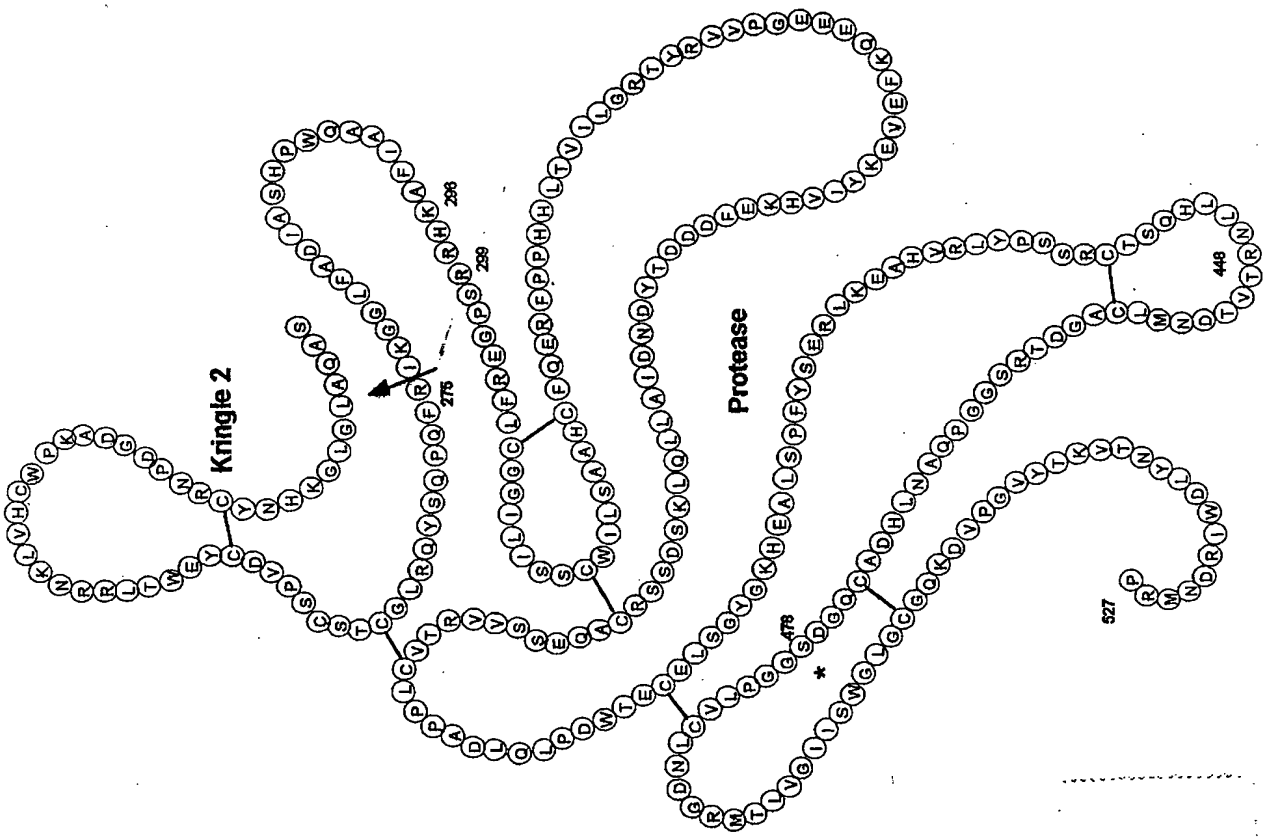


Fig. 14

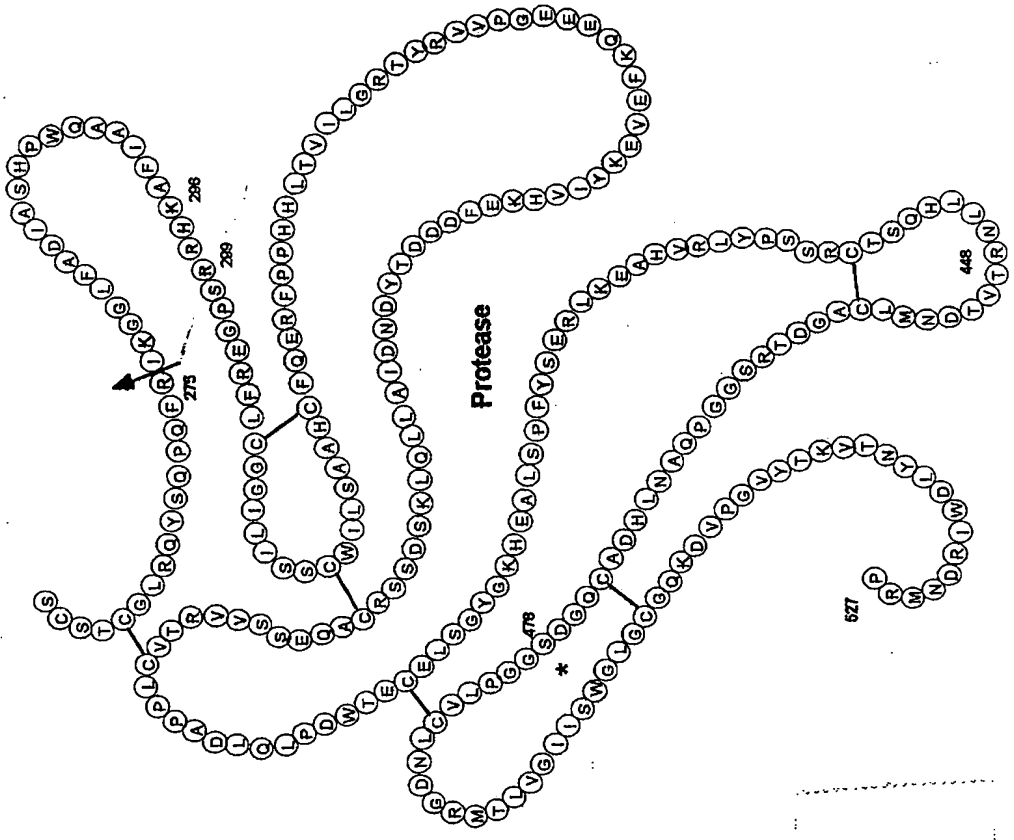


Fig. 15

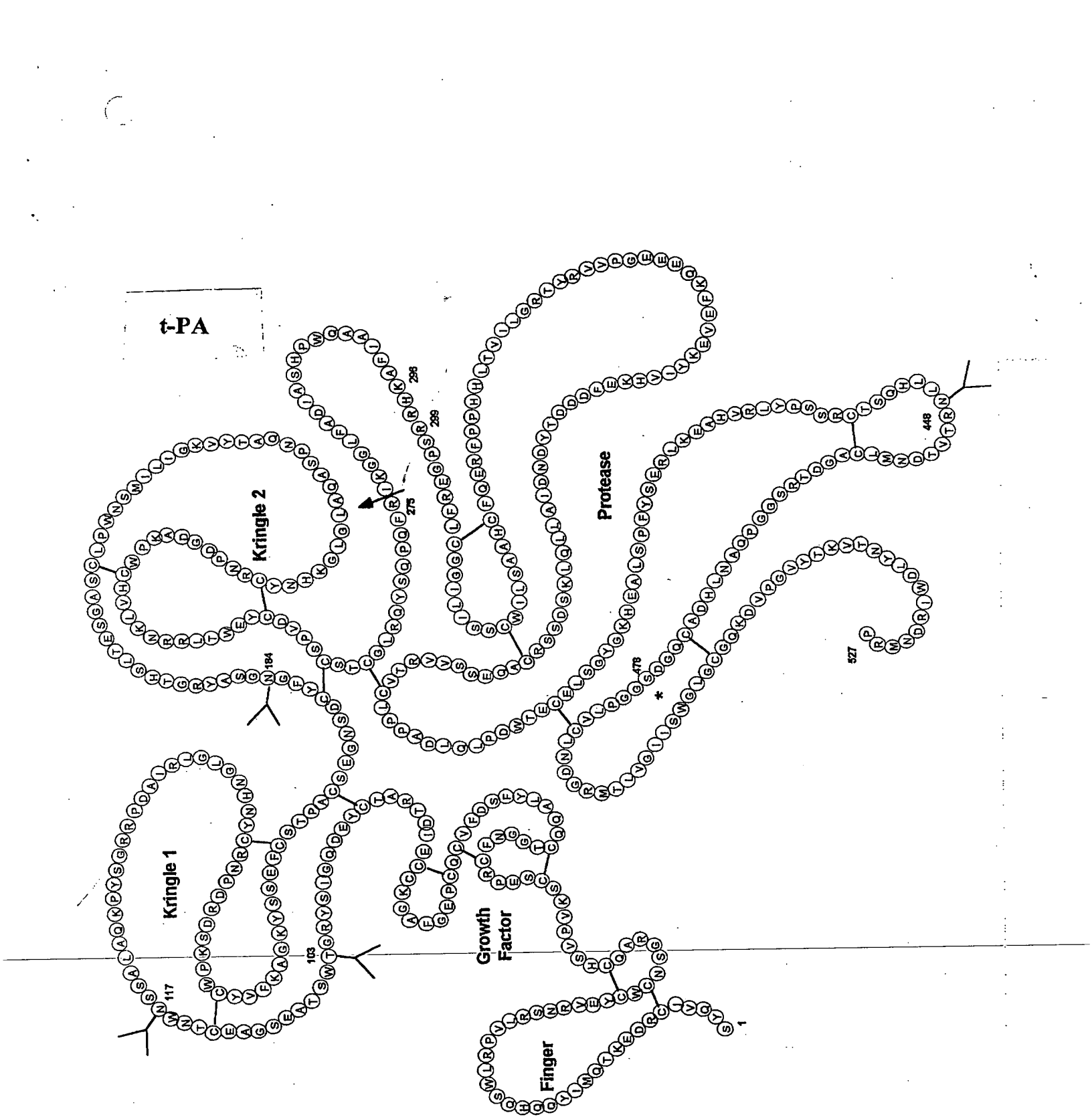

t-PA
Kringle 2
Kringle 1
Growth Factor
Finger
Protease
1
103
117
184
275
296
299
448
478
527

Fig. 16